Role of Transcription-coupled DNA Repair in Susceptibility to Environmental Carcinogenesis

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Susceptibility to environmental carcinogenesis is the consequence of a complex interplay between intrinsic hereditary factors and actual exposures to potential carcinogenic agents. We must learn the nature of these interactions as well as the genetic defects that confer enhanced risk. In some genetic diseases an increased cancer risk correlates with a defect in the repair or replication of damaged DNA. Examples include xeroderma pigmentosum (XP), ataxia telangiectasia, Fanconi's anemia, and Bloom's syndrome. In Cockayne's syndrome the specific defect in transcription-coupled repair (TCR) does not predispose the patients to the sunlight-induced skin cancer characteristic of XP. The demonstration of TCR in the XP129 partial revertant of XP-A cells indicates that ultraviolet (UV) resistance correlates with repair of cyclobutane pyrimidine dimers in active genes. Repair measured as an average over the genome can be misleading, and it is necessary to consider genomic locations of DNA damage and repair for a meaningful assessment of the biological importance of particular DNA lesions. Mutations in the p53 tumor suppressor gene are found in many human tumors. TCR accounts for the resulting mutational spectra in the p53 gene in certain tumors. Li-Fraumeni syndrome fibroblasts expressing only mutant p53 are more UV-resistant and exhibit less UV-induced apoptosis than normal human cells or heterozygotes for mutations in only one allele of p53. The p53-defective cells are deficient in global excision repair capacity but have retained TCR. The loss of p53 function may lead to greater genomic instability by reducing the efficiency of global DNA repair while cellular resistance may be assured through the operation of TCR and the elimination of apoptosis. — Environ Health Perspect 104(Suppl 3):547–551 (1996)

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Introduction

The biological consequences of unrepaired or misrepaired DNA damage depend upon the precise locations of the lesions. DNA lesions at specific sites in the mammalian genome can lead to mutation, recombination, gene amplification, translocation, and other chromosomal abnormalities. These changes in turn may result in malignant transformation, faulty differentiation patterns, or cell death. Thus, it has become clear that damage to DNA at particular loci can cause activation of the protooncogenes and inactivation of tumor suppressor genes that may be implicated in subsequent tumorigenesis (*1*). An increased incidence of neoplasia is correlated with a defect in the repair or replication of damaged DNA in some human genetic diseases. Examples of such hereditary disorders include xeroderma pigmentosum (XP), ataxia telangiectasia (AT), Fanconi's anemia (FA), and Bloom's syndrome (BS) but not Cockayne's syndrome (CS). In the cases of XP and CS, the cultured cells derived from the patients are hypersensitive to short wavelength ultraviolet light (UV), but only for XP does the sunlight sensitivity lead to multiple skin cancers (*2*). In Li-Fraumeni syndrome (LFS), the patients inherit a germ-line defect in one allele of the p53 tumor suppressor gene, resulting in increased neoplastic potential. The loss of the second allele and consequent loss of functional p53 has profound consequences (*3*), including excision repair deficiency in the overall genome as discussed below.

Until recently there has been little information on the fine structure of DNA lesions and their repair in the mammalian genome because the available methodology did not provide such information. Hoping to understand some of the biological phenomena that result from DNA-damaging treatments, my colleagues and I have focused our research in the past decade upon the intragenomic fine structure of DNA damage processing in mammalian cells. It was of particular interest to understand why cellular sensitivity to agents such as UV does not always correlate with measured efficiencies of DNA repair. A classic example is the comparison of the UV sensitivities of human versus rodent cells and their corresponding DNA repair efficiencies. While human and mouse fibroblasts in culture exhibit similar UV survival characteristics, their repair efficiencies are quite different. Within 24 hr after a low UV dose, human cells remove most of the cyclobutane pyrimidine dimers (CPD), the predominant lesion produced, while the rodent cells remove less than 20% (*4*). The rodent cells may achieve high survival by selective repair of their essential active genes in spite of their low overall repair efficiencies. Experiments that document preferential DNA repair in active genes support this explanation (*5–9*).

If one considers the various functional domains of the genome and the potential consequences of unrepaired damage in those regions, actively transcribed genes would appear to be particularly at risk. It is well known that bulky DNA lesions such as CPD pose blocks to the process of transcription *in vivo* and *in vitro* (*10,11*). Incomplete RNA transcripts are produced

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Abbreviations used: AT, ataxia telangiectasia; CHO, Chinese hamster ovary; CPD, cyclobutane pyrimidine dimer(s): CS, Cockayne's syndrome; FA, Fanconi's anemia; LFS, Li-Fraumeni syndrome; TCR, transcription-coupled repair; TEV, T4 endonuclease V; UV, ultraviolet; XP, xeroderma pigmentosum; XP-C, xeroderma pigmentosum complementation group C.

from a gene containing one or more CPD in its transcribed strand. Persisting damage in silent genomic domains may be of less consequence unless it results in accumulating errors in genes that might be expressed at a later stage in the cellular history. Of course gross chromosomal rearrangements resulting from such damage may also seriously affect cellular growth regulation. Damage near replication origins could either block initiation of replication at those origins or distort the cell cycle control of the initiation of replication, possibly resulting in gene amplification. Damage encountered by replication forks may have quite different consequences depending upon whether the lesions are in the leading or the lagging DNA strand. While some types of damage such as interstrand cross-linkage would block replication by preventing parental strand separation, others like O^6 alkylation on guanine may simply alter the coding properties at the site of chain growth. To understand in detail the role of DNA function on biological consequences of damage, it is necessary to measure the lesions and their repair in specific DNA sequences within relevant genomic domains.

Methodology

Traditional methods that have been used to measure DNA damage and repair in cells do not reveal intragenomic heterogeneity in the distribution of lesions or repair patches (*5*). To measure damage in different specific DNA sequences within cells, the sequences of interest must either be isolated directly for analysis or, prior to hybridization probing for the sequences of interest, some partitioning scheme must be employed to distinguish those DNA fragments containing one or more lesions from those with no lesions. Using the latter approach, a versatile technique was developed that can be applied to any DNA sequence for which specific hybridization probes are available (*6*).

In the prototype experiments, a Chinese hamster ovary (CHO) cell line carrying a 50-fold amplification of the dihydrofolate reductase (*DHFR*) gene was used to enhance the sensitivity of detection of a restriction fragment within the gene when the appropriately restricted DNA was electrophoresed under denaturing conditions (*6*). The bacteriophage T4 endonuclease V (TEV) was used as a specific nicking agent for CPD because it cuts the damaged DNA strands at the site of each CPD in the duplex DNA but does not respond to other lesions. Any restriction fragment containing one or more CPD is cleaved by the enzyme so the affected strands would not appear in the electrophoretic gel at the position of the intact fragments. The DNA restriction fragment bands, upon Southern transfer to a nylon membrane, were detected by hybridization with ^{32}P-labeled genomic or c-DNA probes. The proportion of fragments free of endonuclease-sensitive sites in each sample (the zero class) was then determined from the ratio of the amount of probe hybridized at the band position of full-length fragments for the TEV-treated and untreated samples. It is important that the DNA to be analyzed does not include replicated DNA because that would add to the zero class; therefore, density labeling of the replicating DNA with 5-bromodeoxyuridine is used, followed by CsCl equilibrium density gradient centrifugation to separate the unreplicated parental DNA from hybrid daughter DNA. In principle, any type of damage for which specific strand nicking at lesion sites can be accomplished may be quantified in this way. Thus, the UVRABC repair endonuclease enzyme complex from *Escherichia coli* has been used to quantify bulky lesions produced by *cis*-platinum, psoralen, and 4-nitroquinoline oxide as well as UV (*12*). It has also been used to quantify aminofluorene adduct formation and repair in specific DNA sequences (*13*). To investigate the repair of methylated bases, a quantitative method was developed that involves treating the DNA with an appropriate restriction enzyme and then heating it to release *N*-methylpurines (*14*). One portion of each sample serving as control is heated in the presence of methoxyamine to reduce the apurinic sites and protect them from subsequent alkaline degradation. Following alkaline hydrolysis, electrophoresis, transfer to a membrane, and probing for the fragment of interest, the ratios of the band intensities of the DNA sample not treated with methoxyamine to its methoxyamine-treated counterpart are calculated to yield the percentage of the restriction fragments containing no alkaline-labile sites. A procedure was also developed for quantifying psoralen photoadducts in specific DNA sequences, based upon the resolution of DNA fragments containing one or more interstrand cross-links (*15*). The cross-links are determined in renaturing neutral gels while the cross-linkable monoadducts are indirectly quantified by a procedure in which the isolated DNA is reirradiated to convert the maximum number of monoadducts to cross-links prior to analysis on neutral gels. These methods have been described in cookbook detail for different cellular systems and applications (*16–18*).

Repair of CPD in Protooncogenes

Protooncogenes are among the most important cellular targets of physical and chemical carcinogens. Thus, the activation of the H-*ras* protooncogene has been shown to be due to DNA damage produced by carcinogens rather than as a secondary consequence of transformation. The removal of DNA lesions from protooncogenes at risk could therefore represent a critical step in the prevention of tumorigenesis.

As a model system, we used UV-irradiated Swiss mouse 3T3 fibroblasts to compare in the same experiment the formation and removal of CPD in two protooncogenes, the actively transcribed *ABL* gene and the transcriptionally silent *MOS* gene (*19*). Confluent cultures were irradiated with a UV dose of 20 J/m^2, and one portion was lysed immediately while the other was incubated for 24 hr to allow repair. The appropriately restricted DNA samples were either treated or mock treated with TEV prior to electrophoresis, Southern transfer, and hybridization with an *ABL* or a MOS probe. Similar CPD frequencies were found in the two genes initially, but there were marked differences by 24 hr. Substantial repair occurred in the *ABL* gene, while little repair was detected in the *MOS* gene. These results lead to the general prediction that more UV-induced mutations should accumulate in the *MOS* region and in other silent domains than in the *ABL* gene in these cells. While one cannot draw conclusions about the specific role of protooncogene repair in relation to tumorigenesis from this study, it provides a paradigm for similar analyses when the genes at risk in a particular tissue are eventually known.

In another model system to study the effect of the transcriptional state of a protooncogene on its ability to be proficiently repaired, we used the HL60 human promyelocytic cell line which can be differentiated *in vitro*, resulting in the transcriptional modulation of a large class of genes. One of these, the *myc* protooncogene, is actively transcribed in the normally growing undifferentiated promyelocytes. Differentiating the HL60 cells into monocyte/macrophages down regulates *myc* gene expression. When *myc* is actively transcribed, nearly 60% of the UV-induced

CPD are removed within 18 hr. However, when *myc* is transcriptionally down regulated, only 15% of the CPD are removed in that period. Thus, not only the extent but also the rate of repair is higher in the actively transcribed gene. During early differentiation, the *myc* gene is regulated by a block to transcript elongation at the 5′ end of the first intron. Our results reveal no significant difference in the rate of CPD removal between the restriction fragments upstream and downstream of this elongation block. Furthermore, both strands of each fragment exhibit similar repair characteristics. In contrast, the constitutively expressed *FMS* gene exhibits proficient removal of CPD in both the differentiated and undifferentiated cells. Since efficient repair of the active *FMS* gene is maintained in the differentiated cells, the loss of repair competence seen in *myc* is more likely associated with its reduced transcriptional activity than with a decrease in the overall repair capacity of the terminally differentiated cells (*20*).

Features of Transcription-coupled Repair

In studies to further elucidate the nature of the preferential repair in active genes, we compared repair in the transcribed and nontranscribed DNA strands in the *DHFR* gene in both CHO and human cells (*21*). A genomic fragment of the *DHFR* gene was cloned into a vector containing two phage promoters oriented in opposing directions so that RNA probes could be produced to quantify the transcribed and nontranscribed strands, respectively. These analyses were then carried out in turn on the same Southern blot to which the nick-translated DNA probe had been hybridized. Although the same frequency of CPD was measured initially in each strand, there was a very significant difference between the efficiency of repair in the two strands. In the CHO cells nearly 80% of the CPD had been removed from the transcribed strand within 4 hr while almost no repair had occurred in the nontranscribed strand. Similar but less dramatic differences were evident from the analysis of strand-specific repair in human cells. These findings have a number of profound consequences. First is the fact that Poisson statistics cannot be applied with validity to analyze repair in an active gene because the two DNA strands do not represent a homogeneous population. Second is the implication for mutagenesis—the prediction is that one strand in a given active gene will be much more subject to mutation than the other. Recent studies in a number of other laboratories have supported this prediction for UV-irradiated or chemical carcinogen-treated mammalian cells.

Transition-coupled repair (TCR) has now been documented in other cellular systems including *E. coli* (*22*) and *Saccharomyces cerevisiae* (*23–25*). In yeast it operates equally well on an expressed gene on a plasmid as on a chromosome (*25*). In eukaryotes it operates on RNA polymerase II-transcribed genes and requires that the polymerase is actively elongating on the template DNA (*24–26*). RNA polymerase I-transcribed ribosomal genes are not subject to TCR (*27,28*). TCR has been found to operate generally and with high efficiency throughout the normal mammalian cell cycle for the *DHFR* gene that is uniformly expressed in all phases of the human cell cycle. This result was obtained following UV irradiation of an asynchronous culture of human fibroblasts using flow cytometry to sort the population into G_1, early middle and late S, and G_2/M phases, thus avoiding artifacts due to stress-induced synchronization protocols (*29*). These results, incidentally, also rule out the possible association of a replication-coupled DNA repair process analogous to TCR. Clearly, repair is as efficient during G_1 and G_2 phases as in S phase for UV-induced damage in human cells.

Repair of Chemical Adducts to DNA in the *DHFR* Gene

In contrast to the results for CPD, little difference in repair rates is seen for *N*-methylpurines in the *DHFR* gene and in a nontranscribed region located downstream from it in CHO cells. There are also no differences in repair between the transcribed and nontranscribed DNA strands within the gene (*30*). Thus, the small glycosylase that initiates excision repair of *N*-methylpurines may have uniform access to the respective strands. The operation of TCR or not on various other chemical adducts has been reviewed by Bohr (*31*).

We have also measured repair of psoralen monoadducts and interstrand cross-linking diadducts in the human *DHFR* gene and have shown that most of the DNA cross-linking but only half of the monoadducts are removed from a 23 Kb transcribed sequence within 24 hr (*15*). More recently we have extended the study to find that cross-links but not psoralen monoadducts are susceptible to TCR in CHO cells (*32*). Efficient replicative bypass of the persisting monoadducts but not the cross-links has also been demonstrated (*15*). It is likely that most bulky lesions in mammalian DNA other than cross-links pose no insurmountable problems for replication *in vivo*, but they must be removed from essential transcribed sequences to maintain cellular viability. Persisting damage in unexpressed regions and silent genes may result in higher levels of mutation or chromosomal alterations in those regions of the genome. These findings have profound implications for mechanisms of mutagenesis and transformation as well as risk assessment in relation to environmental carcinogenesis.

Models to Explain Fine-structure Heterogeneity in DNA Repair

One can consider a number of potential levels of excision-repair enzyme (or enzyme complex) accessibility to particular lesions in mammalian DNA. Control at different levels of chromatin condensation may constrain the repair of different lesions. Thus, while CPD are efficiently repaired overall in human cells, much of the genome in rodent cells appears to be excluded from repair, as noted earlier. It is of interest in that regard that in xeroderma pigmentosum complementation group C (XP-C), there also appears to be a relatively large portion of the genome that is excluded from repair (*33*). The XP-C cells are UV sensitive, unlike rodent cells that are as UV resistant as normal human cells. However, the rodent cells can repair 6-4 pyrimidine pyrimidones and the XP-C cells are deficient in global repair of these important photoproducts. Venema and co-workers (*34*) found that the residual repair capacity in XP-C was highly selective for active genes. Similar results were obtained in our laboratory; we noted very rapid repair in the active β-actin and *DHFR* genes but poor repair in a silent sequence from the inactive X chromosome (*35*). In contrast, the cells from CS patients are selectively deficient in the repair of active genes. Although CS cells are UV sensitive, there is no deficiency in global DNA repair. However, the cells are deficient in the recovery of UV-inhibited RNA synthesis as originally reported by Mayne and Lehmann (*36*). Venema and co-workers (*37*) showed that CS cells are deficient in TCR. Since XP-C patients are unusually susceptible to UV-induced skin cancer but CS patients are not cancer prone; one might

speculate that the repair of the large silent domains of the genome is particularly significant for this particular end point.

It is well established that XP-A is involved in the recognition of DNA lesions, and many XP-A cell lines are totally devoid of repair of CPD or 6-4 pyrimidine-pyrimidone photoproducts. A partial revertant of XP-A, XP129 was shown to be as resistant to UV as normal human cells; 6-4 photoproduct repair was normal but there was no detectable repair of CPD (*38*). The obvious conclusion was that CPD repair was not important to survival. However, we showed that repair of CPD in the transcribed strand of the expressed *DHFR* gene was as efficient as in normal cells (*39*). Thus, the XP129 cells are capable of TCR, and the enhanced UV resistance in XP129 cells is due in part to the repair of CPD in expressed genes. The repair phenotype of X129 is the same as that of rodent cells generally. They are similarly resistant to UV as human cells and they repair 6-4 photoproducts in the overall genome, but they only repair CPD in expressed genes (*39*). The general conclusion is that overall genomic DNA repair measurements can be misleading when assessing the importance of particular lesions to a biological end point.

Detailed models for the mechanism of TCR have recently been reviewed (*40*) and are beyond the scope of this article. All of the seriously considered models invoke the stalled RNA polymerase at a lesion as an antenna to initiate the recruitment of repair enzymes, as originally suggested by Mellon et al. (*8*).

Role of p53 in Tumorigenesis and in DNA Repair

The p53 tumor suppressor gene product is essential for mediating the responses of mammalian cells to DNA-damaging agents. It is involved in regulation of the cell cycle and in controlling the balance between the promotion of survival through DNA repair and the suicidal process of apoptosis. The apoptotic pathway of cell death is probably important in organs in which severely damaged cells must be removed to promote remodeling of the affected tissue. It is less clear how it might be of value to individual cells in a population.

In damaged cells lacking p53, there may be a failure to arrest the cell cycle in G_1 or to initiate the apoptotic pathway of cell death. Thus, the cells may attempt replication of the damaged genome and accumulate mutations that in turn will contribute to genomic instability. This is consistent with the enhanced cancer risk in patients with LFS. We have investigated the effect of mutations in the p53 gene on UV sensitivity and repair of UV-induced DNA damage in primary human fibroblasts from patients with LFS (*41*) that are heterozygous for mutations in one allele of p53 and sublines expressing only mutant p53. Spontaneously immortalized derivatives of the LFS cell lines have been previously described (*42*). The p53-deficient cells are more resistant to UV cytotoxicity and exhibit less UV-induced apoptosis than normal cells or LFS heterozygotes. DNA repair analysis revealed reduced removal of CPD from overall genomic DNA *in vivo* in the p53 mutant cells compared to the p53 heterozygotes or normal cells. However, the p53 mutant cells retained the ability to carry out TCR (*41*). These results suggest that loss of p53 function may lead to greater genomic instability by reducing the efficiency of DNA repair but that cellular resistance to DNA-damaging agents may be enhanced through elimination of apoptosis. It thus becomes understandable why patients defective in p53 would be at increased risk for tumorigenesis.

Risk Assessment

For purposes of risk assessment, it is sometimes adequate to measure parameters that are really indicators of exposure level rather than directly related to the potentially deleterious consequences of the exposure; however, such gross determinations cannot readily take into account important individual variations that may vastly increase the risks. Therefore, it is important to learn what factors are significant in the probability of progression from environmental exposure to an eventual tumor. The binding of carcinogenic compounds in tissues does not always correlate with the tumorigenicity in those tissues, and a very plausible explanation in some instances could be that different genes are at risk in the different tissues because of their unique patterns of gene expression and DNA repair, respectively.

It is now clear that the repairability of damage in mammalian chromatin depends upon the type of lesion, its precise location in the genome, and the functional state of the DNA at that particular site. Information obtained on the processing of damage overall or in one domain of the genome may not be relevant to an understanding of a biological response that is dependent upon damage and repair activity in another domain. Thus, the question of whether protooncogenes are inefficiently repairable domains in the tissues at risk may therefore have significance for risk assessment. Since rodents are used widely in carcinogen testing for human risk assessment, it is imperative that we learn the unique features of DNA damage processing in the respective systems.

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